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Anesthetic efficacy of tricaine methanesulfonate, metomidate and eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque, 1820)

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Abstract

Anesthetic efficacy, plasma cortisol concentration, and two parameters of neutrophil function (oxidative burst and degranulation of primary granules) were compared among three anesthetics in the fathead minnow: tricaine methanesulfonate (MS 222), metomidate hydrochloride (MTMD), and eugenol (EUG). The optimum anesthetic concentration was determined as: MS 222 75 mg L^{-1}, EUG 30 mg L^{-1} and MTMD 4 mg L^{-1}. Handling and crowding stress was induced in fish with (SA) and without (S) anesthetic. Plasma cortisol concentration was measured at 0, 30, 90, and 240 min after stress and found to increase at 30 min post-stress in S and SA MS 222 groups, but not in SA MTMD and SA EUG groups. To test the effects of different anesthetics on neutrophil function, fish were divided into a baseline control group, a group exposed to handling and crowding stress (S) and a stressed anesthetized group (SA). Fish were assayed for neutrophil function before and after stress (24 h, 72 h and 7 days). The degranulation of neutrophil primary granules was measured as exocytosis of myeloperoxidase (MPO) using 3, 3′, 5, 5′-tetramethylbenzidine as a substrate. Degranulation of primary granules was decreased to 60–75% of non-stressed control in stressed and fish treated with MS 222, and was not affected when MTMD and EUG were used. The degranulation of primary granules proved to be a useful assay for measuring the effects of stress on neutrophil function in fish. Eugenol and metomidate prevented stress-induced decrease of neutrophil function while MS 222 did not.

Keywords: Anesthesia; Neutrophils; Degranulation; Stress; Fish; Fathead minnow

1. Introduction

Anesthesia is a valuable tool in aquaculture, wild fish collection and fisheries management. The capture and

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handling procedures usually have strong effects on fish physiology and behavior (Ross and Ross, 1999), and anesthesia may be used to minimize stress or physical damage caused by crowding, capture, handling and release. Furthermore, in research work or veterinary practice there may be additional requirements to render animals unconscious or to alleviate pain without interfering with physiological processes (Brown, 1988; Ross and Ross, 1999; Bowser, 2001). In addition to preventing physical injury, certain anesthetics reduce or block activation of the hypothalamo–pituitary–interrenal (HPI) axis associated with handling stressors (Iwama et al., 1989; Thomas and Robertson, 1991). Failure to suppress stress-induced activation of the HPI axis results in cortisol release which causes various physiological responses to overcome or compensate for the stress. Severe or chronic stress is often associated with poor performance and has long been suspected to cause immunosuppression in cultured fish (Pickering, 1998).

Tricaine methanesulfonate (Finquel, MS 222) is the only Food and Drug Administration (FDA) approved anesthetic for food fish in the United States (Harper, 2003). It recently has been shown that during handling in fish deeply anesthetized with MS 222, the HPI axis is activated and leads to increased plasma cortisol concentration (Small, 2003). Increased cortisol can indicate stress conditions in fish and the biological effects of cortisol include suppression of immune system responses (Ross and Ross, 1999; Webster et al., 2002). Metomidate (DL-1-(1-phenyl-ethyl)-5-(metoxycarbonyl) imidazole hydrochloride) is a rapid-acting nonbarbiturate hypnotic with potential cortisol suppressing properties (Olsen et al., 1995; Small, 2003). Eugenol (2-methoxy-4-(2-propenyl) phenol) is the major component in clove oil (70–90% by weight) and is listed in the FDA category of materials "generally regarded as safe" (Ross and Ross, 1999). Because of its efficacy, low price, no withdrawal period and lack of negative effects on fish feeding, eugenol and *iso*-eugenol have been considered as future anesthetics of choice in the aquaculture industry (Harper, 2003). It has been recently shown that fish anesthetized with eugenol do not have a significant increase in blood cortisol concentrations (Small, 2003). Anesthetic efficacy of metomidate and eugenol, and the effects of anesthesia on plasma cortisol concentration and innate immune function have not been reported in fathead minnows.

The fathead minnow is an important aquaculture fish species extensively used in bait fish industry, toxicology research, and is a significant forage species for larger predator and game fish (Johnson and Finley, 1980; Harlan et al., 1987; APHA, 1998). Techniques for obtaining relatively pure neutrophil populations have been previously reported for this species (Palić et al., 2005a), allowing for investigation of stress effects on neutrophil function.

Neutrophils are an important component of host defense against many bacterial, viral and fungal infections, and the evaluation of neutrophil function is valuable for assessment of the health status of individuals and animal populations (Densen and Mandell, 1990). There is evidence for phagocytic, chemotactic and bactericidal functions in fish neutrophils, an intense respiratory burst, and peroxidase (myeloperoxidase, MPO) activity (Lamas and Ellis, 1994; Rodrigues et al., 2003; Palić et al., 2005a, 2005b). Measuring exocytosis of myeloperoxidase from primary neutrophil granules in vitro is a direct, rapid and quantitative method to assess the degranulation process in neutrophils that has been used in fathead minnows (Menegazzi et al., 1992; Palić et al., 2005b). Stress is known to decrease neutrophil function in mammals and fish (Barton, 2002; Rice and Arkoosh, 2002; Palić et al., 2005b), and a stress-induced increase in cortisol concentration was correlated to a decrease in oxidative burst and phagocytosis in fish (Barton, 2002). Both direct and indirect effects of HPI activation on neutrophil function were reported: direct cortisol effects on neutrophils are based on dose-dependent activation of glucocorticoid and mineralocorticoid receptors, causing different responses in neutrophils, while indirect effects are based on cortisol-induced production of different cytokines in other cell types (macrophages), leading to suppression of neutrophil function (Frank and Roth, 1986; Webster et al., 2002). Impairment of neutrophil degranulation increased susceptibility to diseases in human beings (Densen and Mandell, 1990), but effects of stress, anesthesia, and increase in blood cortisol concentration on degranulation of neutrophil primary granules have not been studied in fish.

The purpose of this study was to determine the efficacy of eugenol, metomidate and tricaine methanesulfonate in adult fathead minnows, to examine whether metomidate and eugenol suppress the plasma cortisol increase associated with handling and crowding stress, and to determine effects of stress and anesthesia on neutrophil function measured as degranulation of primary granules and superoxide production.

2. Materials and methods

2.1. Fish

Adult fathead minnows with an average weight of 3 g were maintained in the Department of Natural Resource

Ecology and Management, Iowa State University, Ames, Iowa, USA. Fish stock was maintained in 300–1000 L tank recirculation system supplied with dechlorinated tap water at 20 °C and fed daily with dried flake food (Aquatox®, Ziegler Bros Inc, PA, USA). Fathead minnows were cared for in accordance with approved Iowa State University animal care guidelines. Water quality parameters during maintenance period and throughout all experiments were measured two times per week: water temperature was 20±1 °C, pH was 8.0± 0.2, dissolved O_2 was 7±1 mg L^{-1}, total ammonia nitrogen (TAN) was <0.5 mg L^{-1} and total nitrite nitrogen (TNN) was below detection limit (HACH spectrophotometer 2000NR). Preliminary experiments were conducted in order to optimize experimental conditions for stress and anesthetic studies. Results of the preliminary experiments are not shown, but were consistent with the results presented here.

2.2. Establishment of anesthetic efficacy

For purposes of this study, the optimum anesthetic dose for general fish handling was established as the minimum dose producing the desired effect of rapid immobility at stage 3 anesthesia, defined as total loss of equilibrium and cessation of locomotion (Schoettger and Julin, 1969), without medullary collapse and with rapid recovery (Bowser, 2001). Tricaine methanesulfonate (MS 222; Finquel®, Argent Chemical Laboratories, Redmond, WA, USA), metomidate hydrochloride (MTMD; Aquacalm®, provided by Syndel International Inc., Vancouver, BC, Canada), and eugenol (EUG; Sigma, St. Louis, MO, USA) were prepared as stock solutions: MS 222 10 g L^{-1} in deionized sterile water, MTMD 100 mg mL^{-1} in deionized sterile water, and EUG 10 mL in 90 mL of absolute ethanol, and were kept at 4 °C in dark until use.

To determine the anesthetic efficacy and optimal dose of MS 222, MTMD, and EUG, the fish were exposed to different concentrations of anesthetics, and time to induction to 3rd degree of anesthesia, recovery time, and survival were measured. A series of concentrations were prepared from stock solutions in 4 L glass beakers filled with 3 L of tank water buffered with sodium bicarbonate to pH of 8.0 and with constant aeration. The final concentrations of anesthetics were: MS 222 (25, 50, 75, 100, 200 mg L^{-1}), MTMD (1, 2, 4, 8, 16, 32 mg L^{-1}), and EUG (10, 20, 30, 40, 80 mg L^{-1}). Ten fish were quickly netted from the stock tank and randomly split in two beakers (five fish each). Fish were exposed to anesthesia for 20 min and moved to 4 L beaker with 3 L aerated tank water without anesthetic for recovery. The percent of fish in stage 3 anesthesia after 3 min of induction, average recovery time for individual fish, and percent of survival at the time of recovery were determined for each concentration. Fish were moved to the stock tank where food was introduced the next morning and behavior was monitored over the next week.

2.3. Plasma cortisol concentration determination

To determine the effects of handling and crowding stress and effects of MS 222, MTMD and EUG on plasma cortisol concentrations, fathead minnows were stocked in a 1000 L recirculation system for 4 weeks, fish were exposed to optimal concentrations of anesthetic and blood samples were collected from non-stressed fish, fish stressed without anesthetic, and stressed with anesthetic.

To determine baseline plasma cortisol concentrations, 60 randomly netted fish were transferred to six 38 L glass tanks (10 fish per tank) filled with stock tank water and power filtered through active carbon pads. Fish were kept in tanks for 7 days before they were anesthetized with either 200 mg L^{-1} MS 222, 32 mg L^{-1} MTMD, or 80 mg L^{-1} EUG, by adding anesthetic directly to randomly selected tanks (two tanks per anesthetic, procedure repeated for total of 4 samples per treatment). Following the rapid induction of anesthesia at these doses, fish were immediately netted, bled from caudal peduncle with a heparinized capillary tube (Fisherbrand®, 220 μL with ammonium heparin, Fisher Scientific, Hampton, NH, USA) and collected blood (100–150 μL, depending on fish size) was transferred to 1 mL heparinized vial (Vacuette® Minicollect®, Greiner Bio-One, NC, USA). In order to obtain enough plasma for the assay, blood from 3–5 fish was pooled in one vial (total of 600–800 μL) prior to centrifugation (20 min, 800 *g*). Plasma (approximately 50% of full blood volume) was gently removed from the heparinized vial, transferred to 0.5 mL storage capped vial, and kept at −80 °C for subsequent cortisol analysis.

Circulating cortisol concentrations after handling and crowding stress, with or without anesthetics, were determined at 30, 90 and 240 min after initiation of the stress. Five fish were quickly randomly netted from the stock tank and transferred (stress initiated) to 4 L beaker with 3 L of tank water buffered with sodium bicarbonate to pH of 8.0, constant aeration, with a previously determined optimal anesthetic dose (75 mg L^{-1} MS 222, 4 mg L^{-1} MTMD, or 30 mg L^{-1} EUG) or without anesthetic (control). In beakers treated with anesthetic, rapid induction of anesthesia was observed as described above. After 20 min treatment, fish were

returned to 38 L glass tank filled with water from stock tank and aeration and monitored for recovery. After 10 min (30 min after stress initiation), fish were quickly netted, euthanized (500 mg L^{-1} of MS 222) and blood was collected and stored as described above. The procedure was repeated in quadruplicate for all times and treatments/control with a total of 240 fish used (60 fish per treatment/control, 20 fish per time point, 5 fish per sample). Plasma cortisol concentrations were determined by competitive immunoassay cortisol detection kit (Immulite® Cortisol, DPC, Los Angeles, CA) validated for fathead minnow plasma (assay sensitivity 2 ng mL^{-1} in range of 10–500 ng mL^{-1}; interassay variation 5.2–7.4%; intraassay variation 7.2–9.4%; recovery 89–102%).

2.4. Stress and anesthesia effects on neutrophil function

To determine effects of stress and anesthesia on neutrophil function, fish were exposed to handling and crowding stress with and without the optimal dose of anesthetic. During the final stress and anesthetic experiment, each time the fish were exposed to treatment (acute stress procedure with application of anaesthetic), two controls were run in parallel (fish exposed to acute stress procedure without application of anesthetic and non-stressed fish). The treatment was repeated once for each anesthetic (stress and MS 222; stress and eugenol; stress and metomidate), and controls were repeated parallel to each treatment. At the beginning of the experiment (day 0), 252 fish were quickly netted from the stock tank and randomly divided into the following groups: stressed anesthetized group (SA, 108 fish), stressed group (S, 108 fish) and control group (no stress or anesthetic, 36 fish). The SA group was immediately transferred to a 20 L bucket with 10 L tank water buffered with sodium bicarbonate to pH of 8.0, constant aeration and with optimal dose of anesthetic. The third degree of anesthesia was maintained for 20 min, fish were moved to 1000 L recirculation tank supplied with the same water as the stock tank, and within 5 min, 100% of the fish recovered. Fish from the S group were immediately transferred to 20 L bucket filled with 10 L tank water prepared as described above, but without anesthetic. This procedure provided similar (approximately 30 g of fish per liter) handling and crowding conditions compared to fish from SA group. After 20 min of crowding, S group fish were placed in the same tank as SA group, but separated with a net that prevented the mixing of fish. Fish from the control group were immediately killed with an overdose of MS 222 (500 mg L^{-1}), randomly divided into six samples of six fish each and assayed for neutrophil function as day 0. Six samples (randomly selected six fish/sample) from SA group, six samples from S group and six samples from control group (remaining 108 fish from stock tank) were assayed for neutrophil function on days 1, 3 and 7 after treatment. The procedure was repeated for optimal concentrations of tricaine methanesulfonate (SA MS 222), metomidate hydrochloride (SA MTMD) and eugenol (SA EUG).

2.5. Neutrophil separation and functional assays

Kidney tissue was aseptically collected and neutrophils were separated using a previously described technique (Palić et al., 2005a). Briefly, kidneys from six fish were pooled in Hank's balanced salt solution without Ca, Mg and phenol red ($HBSS^{CMF}$, Mediatech-CellGro, AK, USA), homogenized in a 15 mL tissue grinder (Wheaton, USA) and pelleted for 15 min at 250 *g*. The cell pellet was resuspended in $HBSS^{CMF}$ and gently placed over separation medium with a specific gravity of 1.078 g mL^{-1} (Lymphocyte separation medium 1078, Mediatech-CellGro, AK, USA). Gradients were centrifuged for 30 min at 400 *g*, the cells at the interface were removed, washed, resuspended in $HBSS^{CMF}$ and total leukocyte counts and viability (trypan blue dye exclusion) were determined using a Neubauer-ruled hemocytometer (Waterstrat et al., 1988; Andreasen and Latimer, 1989; Palić et al., 2005a). Cell suspensions were adjusted to a standard concentration of 2.5×10^7 cells mL^{-1} and the neutrophil ratio was determined by differential leukocyte counts on Hemacolor (Harleco, EM Science, NJ, USA) stained cytospin preparations of cell isolates (Ellis, 1977; Zinkl et al., 1991; Palić et al., 2005a).

The degranulation of primary granules was assayed as previously described (Palić et al., 2005b). In short, release of myeloperoxidase (MPO) from neutrophils was measured in response to stimulation with calcium ionophore A23187 (CaI, Sigma; 5 μg mL^{-1}) with cytochalasin B (cyto B, Sigma; 5 μg mL^{-1}) in a microtiter plate. Test wells received 75 μL of cyto B and 50 μL of CaI, control (background) wells received 125 μL of HBSS, and total MPO content (lysed cells) wells received 125 μL of cetyltrimethylammonium bromide (lysing solution, CTAB, Sigma; 0.02% in water). 25 μL of cell suspension containing 2.5×10^7 cells mL^{-1} was added to each well, and the plate was incubated at 30 °C for 20 min. After incubation, 50 μL of 3, 3′, 5, 5′-tetramethylbenzidine (TMB, Sigma; 2.5 mM in water) was added, followed immediately with 50 μL of 5 mM H_2O_2. The color change reaction was allowed to proceed for 2 min, and 50 μL of 2 N sulfuric acid was

added to stop the reaction. Test plates were centrifuged at 600 *g* for 15 min, 200 μL of supernatant from each well was transferred to another plate, and optical density (OD) in each well was determined at 405 nM using a microtiter plate spectrophotometer (V-Max, Molecular Devices, USA) with SOFTmax PRO 4.0 software. The percent release of MPO was calculated using the following formula:

$$\%\ \text{release} = [(OD_{stimulated} - OD_{background}) / (OD_{lysed} - OD_{background})] \times 100.$$

A degranulation assay was used to determine effects of stress on neutrophil function as previously described (Palić et al., 2005b). Briefly, the mean percent of neutrophil MPO release from the control group was used as 100% of neutrophil degranulation activity for each day. SA and S group neutrophil degranulation activity were compared to control group activity using the following formula:

$$\%\ \text{of control group neutrophil degranulation activity} = (\%\text{MPO release of SA or } S \text{ group} / \%\ \text{MPO release of control group}) \times 100.$$

Neutrophil oxidative burst was assayed using modifications of a previously described method (Roth and Kaeberle, 1981). Detection of extracellular superoxide was based on reduction of cytochrome *C* (Cyt *C*, Sigma; 490 μg mL^{-1}) with phorbol myristate acetate (PMA, Sigma; 1 μg mL^{-1}) used as stimulant in microtiter plate format, and all samples were tested in duplicate. Briefly, 50 μL of HBSS and PMA were added to each test well, followed by 150 μL of Cyt *C*; non-stimulated test wells received 100 μL of HBSS and 150 μL of Cyt *C*; and background wells received all reagents, but no cells. All test wells received 50 μL of cell suspension containing 2.5×10^7 cells mL^{-1}. The plate was immediately placed in a microtiter plate spectrophotometer (V-Max, Molecular Devices, USA, SOFTmax PRO 4.0 software package) at room temperature, optical density (OD) read every minute for 40 min using two wave lengths ($OD = V_1 - V_2$; $V_1 = 550$ and $V_2 = 650$ nM), and background values were subtracted from entire plate. Total superoxide release was measured as nmol O_2^- produced per 10^6 neutrophils using the following correction formula:

$$O_2^- = [(OD_{av}/2 \times 100)/\%\ N \times 15.87]/1.25.$$

O_2^- = nmol of superoxide produced per 10^6 neutrophils; OD_{av} = average measured optical density; %N = percent of neutrophils in cell suspension; 15.87 = correction factor for transformation of OD value to nmol of O_2^- (Pick and Mizel, 1981); 1.25 = neutrophil dilution factor.

The oxidative burst assay was used to determine the effects of stress on neutrophil function. The mean neutrophil superoxide release from the control group at 20 min was used as 100% of neutrophil burst activity for each day. The 20 min time was used as the average time

Table 1
Efficacy of three different anesthetics for fathead minnows

Tricaine methanesulfonate						
Concentration [mg L^{-1}]	25	50	75[a]	100	200	
Fish in stage 3* [%]	0	40	100	100	100	
Recovery time** [min]	N/A	1.2±0.1[†]	2.4±0.2[†]	6.5±0.7	N/A	
Survival*** [%]	100	100	100	50	0	
Metomidate hydrochloride						
Dose [mg L^{-1}]	1	2	4[a]	8	16	32
Fish in stage 3 [%]	0	0[†]	100	100	100	100
Recovery time [min]	N/A	N/A	3.2±0.5	2.4±0.1[†]	6.7±0.6	10±1.2
Survival [%]	100	100	100	70	80[†]	30
Eugenol						
Dose [mg L^{-1}]	10	20	30[a]	40	80	
Fish in stage 3 [%]	0	30	100	100	100	
Recovery time [min]	N/A	3.1±0.4	4.0±0.4	6.4±0.8	N/A	
Survival [%]	100	100	100	60	0	

Effect of different concentrations of tricaine methanesulfonate, metomidate and eugenol on: *induction of deep surgical anesthesia (stage 3) after 3 min of exposure; **recovery time [mean±SEM] to full equilibrium and swimming behavior after 20 min of exposure; and ***percent survival after 20 min of exposure, was measured. [a]Optimal dose of anesthetic; [†]significantly different from other anesthetics at the same concentration ($P < 0.05$).

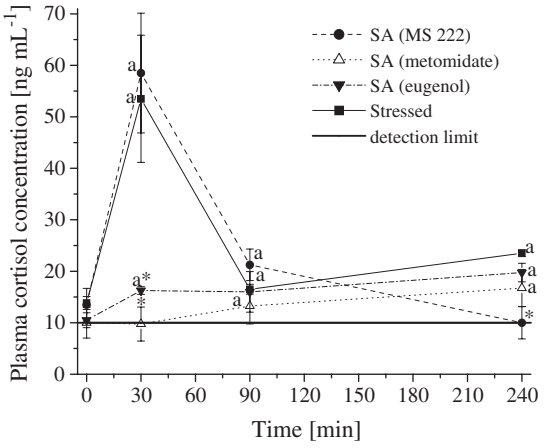

Fig. 1. Plasma cortisol concentrations of fathead minnow monitored for 4 h after 20 min of stress without anesthetic (stressed) and with 75 mg L^{-1} of MS 222 (stressed anesthetized; SA MS 222), 4 mg L^{-1} metomidate (SA metomidate), and 30 mg L^{-1} eugenol (SA eugenol). [a]Significant difference from non-stressed fish (time 0), *significant difference from stressed group ($P<0.05$, $n=4$). Data is presented as mean±SEM.

when O_2^- production reached 95% of maximal recorded value. SA and S group neutrophil burst activity were compared to control group activity using the following formula:

$$\%\ \text{of control group neutrophil burst activity at 20 min} = (O_2^-\ \text{release of SA or } S \text{ at 20 min} / O_2^-\ \text{release of control at 20 min}) \times 100.$$

2.6. Statistical analysis

Data are presented as means±standard error of the mean (SEM) unless otherwise indicated. The differences among groups were analyzed using one- and two-way ANOVA with Dunnett's post-test and Student's *t*-test (GraphPad Prism 3.00, 1999; San Diego, CA). $P<0.05$ was considered significant.

3. Results

3.1. Determination of anesthetic efficacy

Optimal concentrations of the anesthetics are determined to be 75 mg L^{-1} for MS 222, 4 mg L^{-1} for MTMD and 30 mg L^{-1} for EUG (Table 1). At these doses, all fish resumed eating 24 h after treatment and no mortalities occurred during the monitoring period.

3.2. Effect of stress on cortisol concentration

Comparison of circulating plasma cortisol concentrations in fathead minnows exposed to handling and crowding stress with and without MS 222, MTMD, and EUG resulted in significant differences ($P<0.05$) among treatment groups (Fig. 1). Baseline (time 0) cortisol concentrations were similar regardless of the anesthetic used. A significant increase in plasma cortisol concentration was observed at 30 min after treatment for stressed (S; 53 ng mL^{-1}) and stressed anesthetized fish with MS 222 (SA MS 222; 58 ng mL^{-1}). Plasma cortisol concentrations at 30 min in fish from SA MTMD (10 ng mL^{-1}) and SA EUG (16 ng mL^{-1}) groups were significantly lower than in fish from S and SA MS 222 groups. A significant decrease after 30 min in cortisol concentration for S and SA MS 222 groups was observed at 90 and 240 min for S (16 and 23 ng mL^{-1}, respectively) and SA MS 222 (21 and 10 ng mL^{-1}, respectively) groups, while SA MTMD and SA

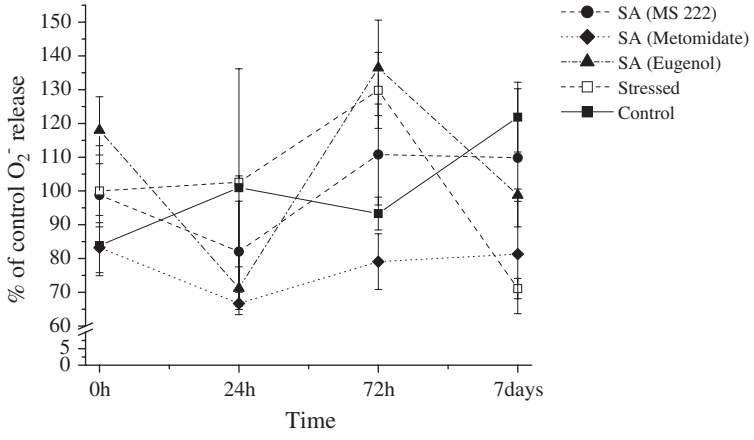

Fig. 2. Effect of stress and optimal dose of anesthetic on respiratory burst in fathead minnow kidney neutrophils measured as superoxide production. No differences were detected between treatments ($P>0.05$). Data is presented as mean±SEM ($n=6$).

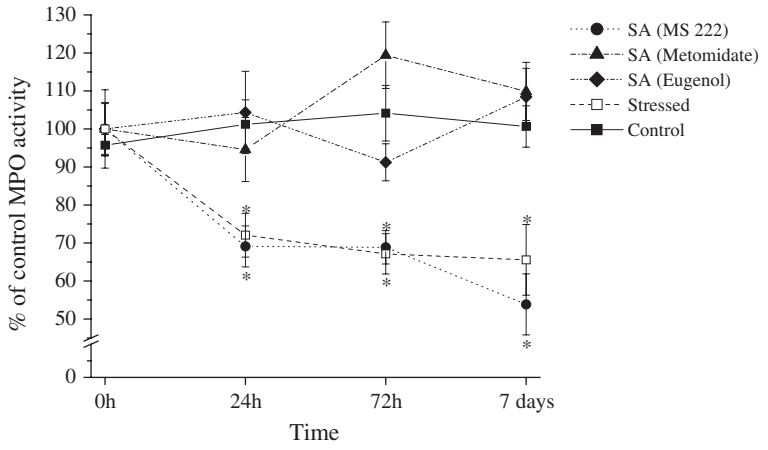

Fig. 3. Effect of handling stress and optimal dose of anesthetics on degranulation of primary granules in fathead minnow kidney neutrophils. *Significantly different from control ($P<0.05$; $n=6$). Data is presented as mean±SEM.

EUG fish maintained cortisol concentration below 20 ng mL^{-1} throughout the sampling period.

3.3. Effect of stress on neutrophil function

Kidney cell suspensions had a mean neutrophil purity of 73.6%±1.8%. The effect of anesthesia on neutrophil oxidative burst is shown in Fig. 2 and effect on neutrophil degranulation is shown in Fig. 3. Production of superoxide anion was similar for all groups at all time points (24 h, 72 h and 7 days post-exposure), and degranulation was significantly reduced at all time points, except time 0, in S and SA MS 222 groups.

A significant decrease in degranulation was detected at 24 h in S and SA MS 222 groups (28% and 31% of control group activity, respectively). A decrease in degranulation for these groups was also observed at 72 h and 7 days after exposure. Neutrophil degranulation in SA MTMD and SA EUG groups was not significantly different from control fish, and varied from 90% to 110% of control group degranulation.

4. Discussion

Stress is inherently involved in aquaculture, research, clinical examination, and all handling procedures in fish, since these may require removal of fish from water. Handling and crowding stress, often combined with poor water quality in intensive aquaculture operations, results in poor performance, disease outbreaks and, in severe cases, increased mortality (Pickering, 1998). Stress can confound experimental results, or further exacerbate disease states (Brown, 1988). Also, there is rising public concern about welfare of fish used in experimental procedures (Fabacher and Little, 2000). Fathead minnows are one of the most often used bait fish, and as such, often exposed to long transport and poor environmental conditions in temporary holding facilities (bait shops or fishermen's bait tanks). In addition, fathead minnows have been extensively used as an animal model in toxicology research and procedures for obtaining EPA permits for chemical use (Russom et al., 1997).

The first part of this study was to evaluate the efficacy of three different anesthetics (tricaine methanesulfonate, MS 222; metomidate hydrochloride, MTMD; and eugenol, EUG) for fathead minnows during stressful procedures, such as handling and crowding, and to establish a minimum dose producing an optimal anesthetic state (Schoettger and Julin, 1969; Ross and Ross, 1999; Bowser, 2001). During the induction of anesthesia, fish treated with MS 222, and EUG, had a longer sedation period prior to complete loss of equilibrium and entering stage 3, compared to MTMD treated fish. This is in agreement with previous reports in catfish, trout and salmon (Gilderhus and Marking, 1987; Olsen et al., 1995; Small, 2003). For the duration of stage 3 anesthesia, MTMD treated fish exhibited small, but rapid, reflex movements if the glass beaker was tapped, fish touched with the airstone, or brushed against other fish in the beaker. Such reflex reactions were not observed in fish treated with MS 222 and EUG. Similar effects of MTMD were reported for catfish, trout, halibut and turbot (Gilderhus and Marking, 1987; Small, 2003), leading to the recommendation that MTMD should not be used as single drug for painful procedures (Hansen et al., 2003). The evaluation of reflex reactions was not an object of this study; however, other studies do not report difficulties in

handling MTMD treated fish (Hansen et al., 2003; Small, 2003). Anesthesia with MS 222 and EUG exhibited a narrow margin of safety when compared to MTMD, having a span of 4 fold from first observable anesthetic effects and 100% survival (50 and 20 mg L^{-1}, respectively) to 0% survival (200 and 80 mg L^{-1}, respectively).

The second part of this study was to evaluate the effects of handling and crowding stress with and without anesthetics on plasma cortisol concentrations. Detection of increased plasma cortisol concentrations has been used as an indicator of stress in fish (Barton, 2002). It has been recognized that some anesthetics can induce a measurable increase in fish cortisol stress responses (Iwama et al., 1989; Thomas and Robertson, 1991; Small, 2003), and it is known that exposure to stressors will cause increase in blood cortisol concentration which may be accompanied by immunosuppression in mammals and fish (Rice et al., 1996; Pickering, 1998; Ortuno et al., 2002a). Anesthetics (e.g. MS 222 and quinaldine) can act to increase cortisol levels even if fish are not subjected to physical stress (Ortuno et al., 2002b; Small, 2003). Other anesthetics (metomidate) are known to inhibit synthesis and release of cortisol (Hansen et al., 2003; Small, 2003), and use of eugenol does not increase cortisol concentrations (Small, 2003). Previous studies found a significant increase (5 fold of control) in cortisol concentrations at 30 min after initiation of stress without use of anesthetic in other fish species (Barton, 2002), and our findings were similar (Fig. 1).

A significant increase (6 fold of control) in cortisol concentrations at 30 min when MS 222 was used, was similar to the increase observed in fish exposed to stress without anesthetic. A significant decrease (1.5 to 2 fold of control) in cortisol concentration for S and SA MS 222 groups was observed at 90 min and was similar at 240 min. In this study, use of MS 222 during stressful procedures did not prevent activation of HPI axis and an increase of the plasma cortisol concentration, and this finding is supported by several published reports (Thomas and Robertson, 1991; Olsen et al., 1995; Pickering, 1998; Small, 2003).

Metomidate treated fish did not exhibit an increase in cortisol concentration at any time point and eugenol treated fish showed only minimal, but significant (1.5 fold of control) increase in cortisol concentrations, that was similar to SA MTMD group for all time points, except 30 min. MTMD was shown to inhibit increases in cortisol concentration in mammals and fish (Limsuwan et al., 1983; Small, 2003), by employing at least two different mechanisms: 1) inhibition of cortisol release by blocking ACTH actions (Thomas and Robertson, 1991; Olsen et al., 1995) and 2) interference with the cortisol synthesis pathway (Vanden Bossche et al., 1984; Wagner et al., 1984). Use of eugenol did not increase plasma cortisol concentrations in catfish (Small, 2003), but the underlying mechanism is unknown. This study demonstrated that handling and crowding stress causes an increase in plasma cortisol concentration in fathead minnows, the use of MS 222 did not prevent a rise in plasma cortisol levels during handling and crowding stress, use of metomidate did not cause a significant increase, and use of eugenol caused a minimal but significant increase at 30 min after the handling and crowding procedure was initiated.

The third part of this study was to evaluate the effects of handling and crowding stress with and without three different anesthetics on the function of fathead minnow kidney neutrophils. The effect of stress on the immune system has been a subject of many studies (Rice and Arkoosh, 2002), but further investigation is needed before a complete understanding of the HPI axis is achieved, including cytokine–neurological interactions (Holland et al., 2002). Cortisol has a significant role in altering immune responses and high doses have been described as immunosuppressive in catfish (Ainsworth et al., 1991). Recent research supports the positive role of low increases in cortisol concentrations as a necessary mechanism to alleviate stress conditions and provide better disease resistance after stress (Webster et al., 2002; Bilodeau et al., 2003). The effects of stress and increased cortisol concentration on neutrophil function have been studied with different exposures, assays, and consequentially have variable results (Ainsworth et al., 1991; Law et al., 2001; Ortuno et al., 2002b). Both direct (through mineralocorticoid and glucocorticoid receptors) and indirect (induction of cytokine release from macrophages) effects of corticosteroids on neutrophils have been reported in mammals (Frank and Roth, 1986; Webster et al., 2002).

Increased levels of corticosteroids can cause a reduction in neutrophil function in mammals, and corticosteroids are well known inhibitors of neutrophil functions such as phagocytosis, migration and oxidative burst (Roth and Kaeberle, 1981; Smith and Lumsden, 1983). Cortisol has been shown to inhibit phagocytosis in leukocytes of common carp and tilapia in vitro (Law et al., 2001). Channel catfish injected with hydrocortisone showed reduced phagocytic activity and bacterial killing, but this effect was not seen in catfish that were exposed to stress (Ainsworth et al., 1991). Seabream showed reduced complement activity and respiratory burst 24 h after multiple stresses (Ortuno et al., 2002b). Use of MS 222 did not significantly affect complement

activity, respiratory burst, or phagocytic activity in seabream (Ortuno et al., 2002a). The production of superoxide, detected by reduction of cytochrome *C* (cyt *C*), has been extensively used for measuring oxidative burst in mammalian neutrophils (Densen and Mandell, 1990). In this study, fathead minnow neutrophils did not show significant differences in oxidative burst between treatment and control groups when measured with the cyt *C* assay. Fathead minnows appear to produce significantly less superoxide anion (3–6 fold) compared to other fish species (unpublished observation), therefore, the cyt *C* assay used in this species may not be sensitive enough to detect changes in the superoxide production due to high variability and low superoxide concentration. It is also possible that, in this species, the superoxide production mechanism is less affected by cortisol or more resilient to stress than the degranulation mechanism.

The assay to measure degranulation of primary granules was originally described to quantitate myeloperoxidase (MPO) in human neutrophils and was used to specifically measure primary granule exocytosis (Menegazzi et al., 1992). Degranulation of primary granules and MPO release is critical for oxidative burst and neutrophil killing (Densen and Mandell, 1990). In fathead minnows, neutrophils are found to be the most abundant granulocyte cell type in kidney isolates, neutrophils have myeloperoxidase activity, (Palić et al., 2005a), and the MPO assay is a direct measurement of neutrophil exocytosis activity (Palić et al., 2005b). Degranulation of primary granules has not been previously used to investigate the effects of stress in fish. We found a significant decrease in degranulation (50–70% of control MPO release) in fish that were exposed to handling and crowding stress with and without MS 222 after 24 h, that remained significantly lower than the controls for the duration of the experiment (7 days). Degranulation in fish treated with metomidate and eugenol was not affected and remained similar to controls for the duration of experiment.

In this study, a rapid, transient increase of cortisol concentration in stressed fish and fish treated with MS 222 was followed by a decrease in degranulation of primary granules detected within 24 h and for as long as 7 days post-treatment. The decrease in degranulation was not observed when a known blocker of cortisol release and synthesis (metomidate) was used, and the increase in cortisol was absent. We hypothesize that the degranulation decrease in stressed fish and fish stressed and anesthetized with MS 222 was caused by observed increase in cortisol concentration, but further research needs to be performed in order to determine if the cortisol acts directly on neutrophils through gluco- and mineralocorticoid receptors, or indirectly through macrophage mediated responses. Recent research argues that low concentrations of cortisol act on leukocytes through a mineralocorticoid receptor with a stronger affinity for cortisol than the glucocorticoid receptor. Only when the cortisol concentration is increased, is the glucocorticoid receptor activated (Webster et al., 2002). Results of this study are in accordance with the suggested model, since in fish treated with eugenol, low increases in cortisol concentrations did not trigger a measurable decrease in degranulation.

5. Conclusion

Optimal anesthetic concentrations for three different anesthetics were established for fathead minnows. The effects of handling and crowding stress with and without anesthesia on plasma cortisol concentration and neutrophil function showed that MS 222 did not prevent increase in plasma cortisol concentration and degranulation, eugenol did not prevent an increase in plasma cortisol concentration, but prevented decrease in degranulation, and metomidate prevented increase in cortisol as well as decrease in neutrophil function. The degranulation of neutrophil primary granules proved to be a useful assay for measuring the effects of stress on neutrophil function in fish.

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